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Activity of Bacteria and Actinomycetes Associated with Mycorrhiza of Pine (*Pinus sylvestris* L.)

By

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Summary

The studies have shown that all bacteria and actinomycetes studied produced auxins in tryptophan containing media. The bacteria were also capable of producing cytokinin-like substances.

All the organisms used produced the vitamins studied. Biotin was produced in smallest and thiamin in largest amounts.

The bacteria and actinomycetes excreted most often glutamic acid, alanine, lysine and valine. Also different organic acids were released by the bacteria studied.

Casamino acids proved to be the most readily oxidized substrate. No bacterial strain exhibited pectolytic activity, but some showed cellulolytic activity.

Introduction

The primary biological fact of the rhizosphere or zone of root influence is the greater number and activity of soil microorganisms in this environment than in the root-free soil.

Microorganisms are known to exert marked influence on growth of plants and are themselves responsive to the environmental conditions imposed by the plant root (Katznelson, 1965; Rovira, 1965).

It is generally accepted that root exudates are the main source of nutrition for the root zone microorganisms (Lochhead, 1940, 1957; Katznelson, 1965; Rovira, 1965). In the mycorrhizosphere also metabolites excreted by the symbiotic fungus are of additional importance for the microbes living in this environment (Rambelli, 1973).

Different organic substances of microbial origin are being considered of importance in affecting plant growth and mycorrhiza formation (Harley, 1948; Katznelson, 1965; Macura, 1971; Slankis, 1973; Haselwandter, 1973; Brown, 1974).

Long ago Harley (1948) suggested that mycorrhizal studies should be done in conjunction with rhizosphere studies. Most researches on microorganisms of the root zone of plants concerned their distribution and identification (Maliszewska and Moreau, 1959; Jensen, 1963; Neal et al., 1968; Rambelli, 1973; Kampert and Strzelczyk, 1975; Dahm and Strzelczyk, 1977; Nioh, 1977). The literature on physiological properties of bacteria and especially of actinomycetes inhabiting the root

zone of forest trees is scarce and fragmentary, although some papers concerning this problem were published (Tribunskaya, 1955; Jensen, 1962; Katznelson et al., 1962; Neal et al., 1964; Rambelli, 1973; Kampert and Strzelczyk, 1984; Strzelczyk and Pokojńska-Burdziej, 1984; Strzelczyk and Różyczki, 1985).

As yet however almost nothing is known about the production of many important organic substances by microorganisms associated with mycorrhizae.

Materials and Methods

Bacteria (pleomorphic types identical or similar to *Arthrobacter globiformis* and spore forming organisms — mainly *Bacillus circulans*) and actinomycetes (*Streptomyces* sp.) were used in this work. The organisms were isolated from soil, rhizosphere and mycorrhizosphere of pine.

The production of the following substances by these microorganisms was studied: auxins, gibberellin- and cytokinin-like substances, B-vitamins, free amino acids and organic acids.

Also the respiratory activity and production of cellulolytic and pectolytic enzymes in bacteria was studied.

Auxins, gibberellin- and cytokinin-like substances were detected by chromatography and bioassay (Strzelczyk and Pokojńska-Burdziej, 1984; Kampert and Strzelczyk, 1984).

Vitamins were determined by bioassay using *Lactobacilli* (ATCC) (Strzelczyk and Różyczki, 1985).

Free amino acids were detected by high voltage electrophoresis and electrochromatography (Różyczki and Strzelczyk, 1985).

Organic acids were separated by high voltage electrophoresis. α -Keto acids were converted into their 2,4-dinitrophenylhydrazones (Różyczki, 1985).

For respiratory studies the manometric technique with the Warburg apparatus was applied (Dahm, 1984).

In studies on cellulase activity reducing sugars were detected colorimetrically. The activity of pectinase was measured viscosimetrically (Dahm et al., 1985).

Results

In tryptophan-free media only 2 bacterial strains produced small amounts of auxins. In media supplemented with this amino acid all bacteria and actinomycetes studied produced auxins (Tab. 1 and 2). The largest amounts of these substances were produced by the mycorrhizosphere bacteria. The highest biological activity was exhibited by substances located at R_f 0.2–0.5. This substance seems to be 3-indoleacetic acid — IAA. Comparison of the culture extracts and pure IAA (using chromatography, UV and Salkowski's test) confirmed the above suggestion. Substances with R_f values 0.6–0.8 and 0.8–1.0 are probably 3-indolebutyric acid and indoleacetic acid nitrile (Bennet-Clark and Kefford, 1953; Stove and Thimann, 1954).

Minute amounts of gibberellin-like substances (with varying R_f values) were detected only in few isolates of the microorganisms studied.

Table 1: Production of auxins and gibberellin-like substances by bacteria isolated from root-free soil and mycorrhizosphere of pine (*Pinus sylvestris* L.)

Bacteria*	Auxins				Gibberellin-like substances	
	Medium without tryptophan		Medium with tryptophan		Total amount of gibberellin-like substances expressed as GA ₃ equivalents	
	Total amount of auxins expressed as IAA equivalents					
	µg/g of dry mass	R _f	µg/g of dry mass	R _f	µg/g of dry mass	R _f
Root-free soil						
<i>Arthrobacter</i> sp. [2]	0	—	272.7	0.3—0.5	trace	0.5—0.6
<i>Bac. circulans</i> [18]	0	—	281.3	0.3—0.5 0.7—0.8	0	—
<i>Bac. circulans</i> [33]	0	—	963.3	0.3—0.5 0.8—1.0	0	—
<i>Arthrobacter</i> sp. [36]	0	—	213.3	0.3—0.5	27.0	0.0—0.1 0.3—0.5
<i>Arthrobacter</i> sp. [51]	0	—	740.7	0.3—0.5	0	—
Mycorrhizosphere						
<i>Arthrobacter</i> sp. [1]	0	—	189.5	0.2—0.5	0	—
<i>Arthrobacter</i> sp. [3]	38.9	0.2—0.4 0.8—1.0	848.8	0.3—0.5 0.7—1.0	0	—
<i>Arthrobacter</i> sp. [19]	0	—	2380.0	0.2—0.4 0.7—0.9	0	—
<i>Arthrobacter</i> sp. [30]	trace	0.3—0.4	1968.8	0.3—0.6 0.7—0.9	0	—

* In paranthesis-strain numbers

The bacteria studied were capable of producing cytokinin-like substances (Tab. 3). The cytokinin-like substances were detected in different column fractions (Tab. 3). The stimulation of the soybean callus was obtained in general with fractions in which either zeatin or zeatin riboside appear.

Table 4 shows the results on B-group vitamins synthesis by the bacteria. All the vitamins studied were produced by these organisms. Thiamin, nicotinic and folic acids were synthesized by the largest number of bacteria. All the vitamins were also produced by the actinomycetes studied. Nicotinic acid and thiamin were the vitamins synthesized by the largest number of isolates. In general a larger number of actinomycetes derived

Table 2: Production of auxins and gibberellin-like substances by actinomycetes isolated from mycorrhizosphere of pine (*Pinus sylvestris* L.)

Strain No.*	Auxins				Gibberellin-like substances	
	Medium without tryptophan		Medium with tryptophan		Total amount of gibberellin-like substances expressed as GA ₃ equivalents	
	Total amount of auxins expressed as IAA equivalents					
	µg/g of dry mass	R _f	µg/g of dry mass	R _f	µg/g of dry mass	R _f
3	0	—	116.0	0.3—0.4	30.0	0.1—0.3 0.4—0.7
9	0	—	145.1	0.2—0.4	0	—
12	0	—	123.8	0.2—0.5	0	—
20	0	—	143.6	0.2—0.4	0	—
34	0	—	237.8	0.2—0.4	0	—
37	0	—	223.0	0.2—0.4	9.0	0.0—0.1
60	0	—	301.2	0.2—0.5	7.8	0.0—0.1

* all strains were *Streptomyces* sp.

from the root zone produced the vitamins than those from the root-free soil. Biotin was produced in smallest and thiamin in largest amounts (Tab. 5).

In studies on free amino acids production it was found that both bacteria and actinomycetes most often excreted glutamic acid, alanine, lysine and valine (Tab. 6 and 7).

The bacteria studied produced also following organic acids: pyruvic, α -ketoglutaric, oxalic, malonic, citric, lactic, succinic, gluconic. The soil bacteria excreted mainly pyruvic and α -ketoglutaric acid. The root zone organisms produced large amounts of pyruvic, gluconic and uronic acids (Tab. 8).

Studies on the metabolic activity of bacteria have revealed that casamino acids proved to be the most readily oxidized substrate. Glucose, sodium acetate, sodium pyruvate and especially starch were less willingly utilized.

Studies on cellulolytic and pectolytic activity have shown that 5 bacterial strains from among 20 used exhibited cellulolytic activity. Almost all the actinomycetes produced endoglucanase in media with carboxymethylcellulose and 7 from among 12 isolates were pectolytic. Detailed data on the respiratory activity as well as the enzymatic activity are not included.

Table 3: Amount of cytokinin-like substances produced by soil, rhizosphere and mycorrhizosphere bacteria

Source of isolation	Strain No.	Bacteria	Amount of cytokinin-like substances [kinetin equivalents in $\mu\text{g}/1.0\text{ g}$ of dry mass of bacteria]	Fraction No.
Soil	2	<i>Arthrobacter</i>	0	
	4	<i>Arthrobacter</i>	0	
	9	<i>Bacillus circulans</i>	0	
	10	<i>Bacillus circulans</i>	0	
	15	<i>Bacillus circulans</i>	0	
	17	<i>Bacillus circulans</i>	4.3	28-34, 72-76, 80-88, 96-104,
	20	<i>Bacillus circulans</i>	30.2	0-8, 24-32, 36-68, 72-104,
	26	<i>Bacillus circulans</i>	66.6	20-24, 28-44, 48-60, 72-76, 80-104,
	30	<i>Bacillus circulans</i>	14.1	68-72, 76-80, 84-90,
Mycorrhizosphere	3	<i>Arthrobacter</i>	4.6	60-68,
	5	<i>Arthrobacter</i>	0	
	13	Gram-negative rod	0	
	19	<i>Arthrobacter</i>	0	
	23	<i>Arthrobacter</i>	8.3	88-92,
	32	<i>Arthrobacter</i>	9.6	28-32, 56-60,
Rhizosphere	3	<i>Arthrobacter</i>	0	
	4	<i>Arthrobacter</i>	6.9	0-8, 24-28, 32-84, 88-92, 100-104
	5	<i>Arthrobacter</i>	8.0	88-96,
	18	Gram-negative rod	6.2	88-92,
	10	Gram-negative rod	0	
	15	<i>Arthrobacter</i>	16.8	48-52, 92-100,

Explanations: Fractions No. 36-60 correspond to zeatin riboside and fractions No. 60-80 to zeatin.

Table 4: Amount of vitamin synthesized by the bacteria (µg/g dry weight)

Vitamins:	Source of isolation:								
	Soil			Rhizosphere			Mycorrhizosphere		
	Number of strains producing the vitamins	Range	Mean	Number of strains producing the vitamins	Range	Mean	Number of strains producing the vitamins	Range	Mean
Biotin	1	0.021	0.001	3	0.011— 0.031	0.003	4	0.001— 0.101	0.011
Folic acid	8	0.062— 0.335	0.088	6	0.048— 1.190	0.126	8	0.017— 0.920	0.092
Nicotinic acid	8	4.128— 60.090	6.982	9	6.009—44.025	8.950	5	3.077— 23.707	3.134
Pantothenic acid	1	110.416	5.521	1	14.852	0.743	3	6.842— 29.476	5.238
Riboflavin	1	4.697	0.235	4	2.148—18.687	2.364	6	1.028— 29.883	5.238
Thiamin	10	1.059—163.351	53.301	8	0.604—74.386	12.186	6	0.588—122.549	10.643
Number of strains studied	20			20			20		

Values indicated in the same lines by the same letter do not differ statistically ($P \leq 0.05$)

Table 5: Amount of vitamins synthesized by the actinomycetes

Quantity of vitamins in µg/g dry weight											
Source of isolation											
Vitamins	Number of strains producing the vitamins	Soil			Number of strains producing the vitamins	Rhizosphere			Number of strains producing the vitamins	Mycorrhizosphere	
		Range		Mean		Range		Mean		Range	Mean
Biotin	14	0.11—	3.46	0.93 ^a	19	0.05—	4.35	1.23 ^a	10	0.22—	3.06
Nicotinic acid	18	75	—703.12	244.03 ^a	20	73.30—	1500	451.08 ^b	18	20.83—	494.31
Pantothenic acid	11	7.18—	720	207.91 ^a	20	122.28—	1935.61	893.81 ^b	17	21.03—	2033.83
Thiamin	16	75.75—	690.78	366.92 ^a	19	340.90—	4233.87	1935.19 ^b	20	103.65—	2571.42
Number of strains studied	20	—			20	—			20	—	

Values indicated in the same lines by the same letter do not differ statistically ($P \leq 0.05$)

Table 6: Bacteria of soil, rhizosphere and mycorrhizosphere synthesizing large amounts of some amino acids

Source of isolation	Number of isolates studied	Amino acids synthesized in large amounts ¹⁾													
		Glu	Ala	Icu	Val	Lys	Ser	Thr	Tyr	X ₂	X ₃	X ₅	X ₇	X ₈	X ₉
Soil	15	11 ²⁾	9	3	4	0	1	0	0	1	3	3	0	1	1
		73.3 ³⁾	60.0	20.0	26.7		6.7			6.7	20.0		6.7	6.7	
Rhizosphere	15	3	5	2	2	2	0	1	0	4	1	0	0	0	0
		20.0	33.3	13.3	13.3	13.3		6.7		26.7	6.7				
Mycorrhizosphere	20	6	17	14	14	4	2	2	1	4	6	0	1	1	0
		30.0	85.0	70.0	70.0	20.0	10.0	10.0	5.0	20.0	30.0		5.0	5.0	
Total	50	20	31	19	20	6	3	3	1	9	10	3	1	2	1

Explanations: ¹⁾ big, strong-coloured spots on the electrochromatogram; ²⁾ number of isolates; ³⁾ per cent

Table 7: Amounts of the amino acids produced by the actinomycetes studied ($\mu\text{g}/\text{mg}$ dry mass)¹⁾

Amino acids:	Source of isolation:		
	Soil	Rhizosphere	Mycorrhizosphere
Glutamic acid	1.5030 ^a ±0.2370	43.4840 ^b ±26.0100	34.1546 ^b ±9.9843
Aspartic acid	0.2080 ^a ±0.0782	0.9130 ^a ±0.6310	0.5806 ^a ±0.1391
Alanine	7.1380 ^a ±3.8330	26.4190 ^a ±17.8330	15.8594 ^a ±9.1554
Lysine	1.0850 ^a ±0.4800	5.5380 ^b ±2.4340	2.4359 ^b ±0.3111
Tryptophan	0.1619 ^a ±0.0251	0.2701 ^a ±0.0747	0.2136 ^a ±0.0397
Number of strains studied	10	10	10

Explanations: ¹⁾ mean values ± standard error; values indicated in the same lines by the same letter do not differ statistically ($P \leq 0.05$)

Table 8: Amounts of some organic acids produced by the bacteria ($\mu\text{g}/\text{mg}$ dry mass)¹⁾

Acids	Source of isolation:		
	Soil	Rhizosphere	Mycorrhizosphere
α = Keto acids			
Pyruvic	27.55 ± 12.21	1032.20 ± 535.90	58.24 ± 40.81
α -Ketoglutaric	15.28 ± 6.24	0.72 ± 0.72	1.71 ± 0.80
Number of strains studied	10	4	5
Sugar acids			
Gluconic	²⁾ —	3475.44 ± 1898.00	5399.19 ± 571.82
Uronic (γ)	—	906.44 ± 73.95	1052.97 ± 534.62
Number of strains studied	—	3	9

Explanations: ¹⁾ mean values ± standard error; ²⁾ not studied

Discussion

As mentioned in the introduction microorganisms may stimulate or inhibit growth of mycorrhizal fungi and mycorrhiza formation.

Shemakhanova (1962) found that *Trichoderma lignorum*, *Azotobacter chroococcum* and fluorescent bacteria supported formation of mycorrhiza of pine. Also Malyskin (1955) found that *Azotobacter* and *Trichoderma* stimulated mycorrhiza formation. This action is attributed to the extracellular substances they release. Vedenyapina (1955) is of the opinion that the effect of *Azotobacter* in mycorrhiza formation does not depend on peptolytic activity or nitrogen fixation but rather on the

production of vitamins which stimulate the mycorrhizal fungi. However Vozyakovskaya and Ryzhkova (1955) are of the opinion that there are facts indicating the dependence of mycorrhiza formation upon the presence of certain microbial associations. According to these authors certain microbial groups living in the mycorrhizosphere facilitate the penetration of the symbiotic fungus into the root tissues of the host.

On the other hand also detrimental effects of associated microorganisms on mycorrhiza formation have been reported. Brian et al. (1945) found that the failure of *Boletus bovinus* to form mycorrhiza is caused by the antibiotic gliotoxin produced by certain penicillia. Antagonism and competition are suggested by Bowen and Theodorou (1979) as mechanisms for depression of mycorrhizal fungi.

Thus it seems that the metabolites excreted by microorganisms associated with mycorrhizae may be of great importance in formation and maintenance of mycorrhiza. It is accepted that plant growth regulators like auxins and cytokinins stimulate or are essential in mycorrhiza formation (Moser, 1959; Slankis, 1973; Crafts and Miller, 1974; Tomaszewski and Wojciechowska, 1974). The role of gibberellins in mycorrhiza formation is to our knowledge not known. However they may act in conjunction with other plant growth regulators. This however requires detailed studies.

Development of mycorrhizal fungi depends especially on thiamin, less on biotin (Palmer, 1971). The associated organisms studied produced in largest amounts thiamin. According to Moser (1959) the main source of vitamins for the mycorrhizal fungi are likely to be rather the rhizosphere microorganisms than the plant itself.

Some mycorrhizal fungi like *Cenococcum graniforme* and *Tricholoma* sp. were found by Melin (1953) to require certain amino acids. According to Fries (1976) some amino acids stimulate germination of basidiospores of *Suillus luteus*. Asparagine prevented germination of basidiospores of this fungus.

The studies on physiology of microorganisms associated with mycorrhiza seem to have scientific and practical values. They are of importance in better understanding the interrelationships between the symbiotic fungus and associated microorganisms. They also may have practical application during inoculation of sterile or semi sterile forest soils or woody plants seedlings.

Zusammenfassung

Aktivität von mit der Mykorrhiza der Kiefer (*Pinus silvestris* L.) assoziierten Bakterien und Actinomyceten

Die Untersuchungen haben gezeigt, daß alle geprüften Bakterien und Actinomyceten in Tryptophan-haltigen Medien Auxin produzieren. Darüber hinaus konnten die Bakterien auch Cytokinin-ähnliche Substanzen produzieren. Alle Organismen bildeten Vitamine. Hierbei war die Biotin-Produktion am geringsten und die Thiamin-Produktion am stärksten. Bakterien und Actinomyceten schieden oft Glutaminsäure; Alanin, Lysin und Valin aus. Daneben wurden durch die untersuchten Bakterien auch verschiedene organische Säuren freigesetzt. Die Casamminosäuren erwiesen sich als die am vollständigsten oxidierten Substanzen. Kein Bakterium zeigte pektolytische, wenige zeigten zellolytische Aktivität.

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